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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : C12N 15/00	A2	(11) International Publication Number: WO 99/24559
		(43) International Publication Date: 20 May 1999 (20.05.99)

(21) International Application Number: PCT/GB98/03385

(22) International Filing Date: 11 November 1998 (11.11.98)

(30) Priority Data:
9723825.7   11 November 1997 (11.11.97)   GB

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: NUCLEAR TARGETING BY MEANS OF BACTERIAL PROTEINS

(57) Abstract

A nuclear delivery construct comprises: (i) protein H or a fragment or derivative thereof that is capable of being targeted to the nucleus of a eukaryotic cell; and associated therewith (ii) one or more other components whose targeting to the nucleus of the eukaryotic cell is desired.

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NUCLEAR TARGETING BY
MEANS OF BACTERIAL PROTEINS

FIELD OF THE INVENTION

5

The present invention relates to nuclear targeting by
means of bacterial proteins. It is based on the finding
that protein H, which can be derived from *Streptococcus
pyogenes*, is targeted to the nuclei of eukaryotic cells,

10   specifically lymphocytes. The present invention therefore
relates to nuclear targeting constructs comprising
protein H, or a fragment or derivative thereof,
associated with another component whose targeting to the
nucleus is desired. It also relates to uses of such

15   nuclear targeting of such constructs, and to
pharmaceutical compositions comprising them.

BACKGROUND TO THE INVENTION

20   Protein H can be obtained from *Streptococcus pyogenes*, as
described in EP-A-0 371, 199 and WO 91/19740. These
publications also provide the amino acid sequence of
protein H from *Streptococcus pyogenes* and the sequence of
the DNA encoding it. Protein H has a characteristic

25   spectrum of immunoglobulin-binding properties, as
described in EP-A-0 371,199 and it is also capable of
binding albumin (WO 91/19740). In WO 91/19740, a number
of regions within protein H were identified, and
designated S, A, B, C1, C2, C3 and D regions. Albumin-

30   binding activity was found to be located in the C and/or
D regions.

SUMMARY OF THE INVENTION

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We have now found that protein H has a further, and
unexpected, property. When biotinylated protein H was
incubated with T-lymphocytes (Jurkat cells), it was found
that protein H was targeted to the cell nucleus. We have
5   also observed the same phenomenon in B-lymphocytes.

In the cell, protein H was found to interact with actin,
and with nucleophosmin/B23, a protein known to shuttle
between the nucleus and cytoplasm. In the nucleus itself,
10   protein H was found to interact additionally with the
nuclear proteins SET and hnRNP A2/B1, resulting in
nuclear accumulation of protein H. We believe that
protein H penetrates the cell membrane, becomes
associated with nucleophosmin/B23 in the cytoplasm and is
15   transported across the nuclear membrane into the nucleus,
where it interacts with SET and hnRNP. As protein H
interacts with actin, the actin cytoskeleton may be
involved in transporting protein H from the inside of the
cell membrane into the cytoplasm, where it becomes
20   associated with NPM/B23. However, the possibility that
protein H simply diffuses through the cytoplasm cannot be
excluded.

The finding that protein H is targeted to the nucleus was
25   surprising in view of the previously known properties of
protein H. Previously, protein H has been known as an
immunoglobulin-binding protein, located at the surface of
the Streptococcus bacterium, where it protects the
bacterium by blocking complement activation at the
30   bacterial cell surface by means of its interaction with
the Fc region of IgG. It is therefore surprising that it
is also targeted to the cell nucleus, where it may effect
a further virulence function on behalf of the
Streptococcus bacterium. By contrast, protein A, an
35   immunoglobulin-binding bacterial cell surface molecule

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derived from *Staphlyococcus aureus*, showed no nuclear
accumulation. Proteins A and H are functionally similar
in that both bind to the same site in the Fc region of
IgG. The fact that protein H showed nuclear accumulation
5   where protein A did not is therefore all the more
surprising.

Based on our findings, protein H can be used to target
other molecules to the nucleus. For example, protein H
10   can be used to target drugs to the nucleus, or to target
nucleic acids to the nucleus for the purpose of gene
therapy.

We have also found that protein H has a cytostatic effect
15   when targeted to the nucleus. In certain situations, for
example where a drug is delivered to a proliferating
cancer cell for the purposes of treating the cancer, this
cytostatic effect will complement the effects of the
associated drug. In other situations, it may be desirable
20   to use fragments or derivatives of protein H that do not
exhibit the cytostatic effect but are nevertheless
capable of being targeted to the nucleus.

The invention accordingly provides a nuclear delivery
25   construct comprising:

(i)   protein H or a fragment or derivative thereof that
       is capable of being targeted to the nucleus of a
       eukaryotic cell; and

30

       associated therewith

(ii)   one or more other components whose targeting to the
       nucleus of the eukaryotic cell is desired.

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The invention also provides a pharmaceutical composition
comprising such a nuclear delivery construct and a
pharmaceutically acceptable carrier.

5   The invention also provides such a nuclear delivery
construct for use in a method of treatment of the human
or animal body, or for use in a method of diagnosis.

The invention also provides use of protein H or a
10 fragment or derivative thereof that is capable of being
targeted to the nucleus of a eukaryotic cell in the
manufacture of a medicament for the treatment of the
human or animal body in which such a construct is
targeted to the nucleus of a eukaryotic cell.

15

The invention also provides use of such a nuclear
delivery construct in the manufacture of a medicament for
the treatment of disease of the human or animal body in
which the construct as defined in claim A is targeted to
20 the nucleus of a eukaryotic cell.

The invention also provides a method of treating a
disease of the human or animal body by targeting such a
construct to the nucleus of a eukaryotic cell, said
25 method comprising administering an effective amount of
the construct to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

30 **Figure 1.** Binding of protein H to the surface of human
peripheral blood lymphocytes and the human Jurkat T cell
line determined by FACS analysis.

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Figure 2. Uptake and nuclear accumulation of protein H in
Jurkat T cells. Depiction of Jurkat T cells incubated
with proteins A or H, cytospinned and stained with
FITC-avidin. Protein H (A, B) shows nuclear accumulation,
protein A (C) does not.

Figure 3. Protein H interacts with nucleophosmin. (A)
Results of FPLC on mono-Q column of Jurkat T cells
digested with papain and solubilised. (B) Results of
radiolabelled pool 85-87 material from (A) being run on
protein A-Sepharose (left)and the pooled fractions of the
run-through peak from protein A-Sepharose being subjected
to a protein H-Sepharose column (right). (C)
Identification of NPM in pool 85-87 material from (A).
(D) Comparison of SDS PAGE of *in vitro* translated and
^{35}S-methionine-labelled NPM peptides (left) and SDS-PAGE
of the same peptides when applied to protein H-Sepharose.
(E) Mapping of the NPM-binding region of protein H by
competitive inhibition.

Figure 4. Identification of nuclear proteins interacting
with protein H. SDS-PAGE gels identifying NPM, protein
SET and hnRNP A2/B1.

Figure 5. Analysis of the binding of protein H to
nucleophosmin and nuclear proteins. (A) Overlay plots of
binding of proteins A and H to immobilised NPM (left) or
nuclear proteins (right) using plasmon resonance
spectroscopy. (B) Competitive inhibition of the binding
of ^{125}I-labelled NPM to protein H-Sepharose with different
amounts of unlabeled NPM.

Figure 6. Nuclear uptake and cytostatic effect of protein
H. (A) SDS-PAGE gels of purified murine B cells incubated

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with LPS and protein H for 24 hours stained with
Coomassie (left) or blotted to a PVDF membrane (right),
with the blotted membrane probed with an anti-protein H
antiserum, followed by peroxidase-conjugated protein A
5   and developed with ECL.(B) Inhibition of proliferation of
murine splenic B cells in response to proteins H and A.

Figure 7. Schematic representation of protein H.

10   **DETAILED DESCRIPTION OF THE INVENTION**

Protein H

In WO 91/19740, protein H was characterised as having the
15   following domains, from N-terminal to C-terminal: S, A,
B, C1, C2, C3 and D. The S domain is a signal peptide
which, in nature, is cleaved from the remaining domains
before the mature protein(domains A, B, C1, C2, C3 and D)
is translocated to and inserted into the bacterial cell
20   wall.

In the Examples below, a shorter version of protein H is
used. This lacks the 41 amino acids of the N-terminal S
domain and is also truncated by 30 amino acids at the C-
25   terminus. This version of protein H was produced in *E.
Coli*. It is similar to the version of protein H which, in
nature, is released from the cell surface of the
Streptococcus bacterium by the action of a cysteine
protease. The version released by the protease also lacks
30   the S domain and is also truncated at the C-terminus. It
is slightly shorter than the version produced in *E. Coli*,
however.

Herein, unless otherwise stated, the term "protein H"

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means either:

(i) protein H incorporating the signal peptide, as
defined in WO 91/19740 and having S, A, B, C1, C2, C3 and
5   D domains and a length of 376 amino acids; or

(ii) mature protein H lacking the S domain and having a
length of 335 amino acids; or

10   (iii) protein H as produced in *E. Coli*, lacking the S
domain and truncated by 30 amino acids at the C-terminus,
and having a length of 305 amino acids; or

(iv) protein H as cleaved from the *Streptococcus* cell
15   surface by the cysteine protease, lacking the S domain
and truncated at the N-terminus by a number of amino
acids. The precise length of this version of protein H is
not yet known, but its molecular mass suggests that the
N-terminal truncation is in the order of 50 amino acids,
20   such that it has a length of approximately 285 amino
acids. Thus, this version of protein H may, for example
have an N-terminal truncation of from 35 to 45, 45 to 50,
50 to 55 or 55 to 65 amino acids, giving it a length,
respectively, of 270 to 280, 280 to 285, 285 to 290 or
25   290 to 300 amino acids.

The full 376 amino acid sequence is given below (see
Sequence information section) as SEQ ID No. 6, together
with the coding DNA sequence (SEQ ID No. 5). Amino acid
30   No. 1 represents the beginning of the A domain and the
boundaries of each region and each version of protein H
are indicated on the sequence. In other words, amino acid
No. 1 is the first amino acid of the mature protein
(version (ii) of protein H as defined above). The
35   numbering differs from that used in WO 91/19740 in that,

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in WO 91/19740, amino acid No. 1 is the first amino acid
of the S domain, which is a signal peptide that is absent
from the mature protein.

5   Protein H can be obtained by the methods described in EP-
       A-0 371, 199 and WO 91/19740, and can also be produced
       using the methods of Åkesson et al, 1990; and Frick et
       al, 1994, in combination with the methods described in
       the Examples. Any of the above-mentioned versions of
10   protein H can also be synthesised by recombinant means,
       based on the sequences given herein and using standard
       techniques known in the art (as exemplified, for example,
       by Sambrook et al, 1989, Molecular Cloning: A Laboratory
       Manual). The same applies to fragments and derivatives of
15   protein H as defined herein. Similarly, protein H and
       fragments/derivatives thereof can be prepared
       synthetically by techniques of peptide synthesis already
       known in the art. This applies especially to fragments of
       protein H.
20

Nuclear targeting

       Based on the experimental results presented herein,
       protein H is capable of being targeted to the nuclei of
25   eukaryotic cells. Targeting has been demonstrated in T-
       lymphocytes (Jurkat cells) and B-lymphocytes (Bjab
       cells).

       For the purposes of the invention, protein H and its
30   fragments and derivatives are capable of being targeted
       to the nuclei of eukaryotic cells. A person of skill in
       the art can determine whether or not any given fragment
       or derivative is capable of being so targeted in any
       given cell type using techniques based on those described
35   in the Examples. For example, the fragment or derivative

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can be biotinylated, then incubated with the cells in
question. The intracellular distribution of the
biotinylated fragment or derivative can then be
determined, for example using FITC-Avidin in conjunction
5   with immunofluorescence microscopy. A person of skill in
the art will also be able to devise additional
methodologies to determine whether or nor protein H or a
fragment or derivative thereof is capable of being
targeted to the nucleus of any given cell type.
10

Nuclear localisation has been demonstrated in T-
lymphocytes (Jurkat cells) and B-lymphocytes (Bjab
cells). Preferably, constructs of the invention effect
targeting to T-lymphocytes and/or B-lymphocytes. It is
15   not yet clear whether protein H is capable of being
targeted to the nuclei of all eukaryotic cell types or
whether it is selectively targeted to the nuclei of
certain cell types, or whether it is targeted to the
nuclei of all cell types to some extent but more strongly
20   in certain cell types. However, this can readily be
determined by a person of skill in the art as described
above.

In accordance with the invention, it is preferred that
25   protein H and its fragments/derivatives are capable of
interacting with nucleophosmin/B23. It is also preferred
that protein H and its fragments/derivatives are capable
of interacting with actin. It is also preferred that
protein H and its fragments/derivatives are capable of
30   interacting with the nuclear protein SET. It is also
preferred that protein H and its fragments/derivatives
are capable of interacting with the nuclear protein hnRNP
A2/B1. Optionally, protein H and its
fragments/derivatives may be capable of interacting with
35   further cytoplasmic and/or nuclear proteins, for example

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other hNRNPs, or transcription factors. A person of skill
in the art can determine whether or not a given
fragment/derivative does interact with any of the above-
mentioned proteins using techniques based on those of the
5   Examples.

Although it is not yet certain whether protein H is
capable of being targeted to the nuclei of all eukaryotic
cell types or whether it is selectively targeted to the
10   nuclei of some cell types, it is possible to identify
some preferred cell types to which protein H and its
fragments/derivatives can be used to target associated
components in nuclear delivery constructs.

15   Nucleophosmin (NPM)/B23 is known to act to shuttle
proteins between the cytoplasm and the nucleus. It is
ubiquitously expressed but it is up-regulated in certain
cell types. In particular, NPM/B23 is more abundant in
tumour and proliferating cells than in resting cells. It
20   is reasonable to predict that proliferating and tumour
cells, having a larger available pool of NPM/B23, will be
more effective in translocating protein H and its
fragments or derivatives into the nucleus. Thus,
according to the invention, it is preferred to use
25   protein H and its fragments/derivatives to target
associated components to cells in which NPM/B23 is up-
regulated.

Preferred cell types in which protein H and its
30   fragments/derivatives can be used to target associated
components to the nucleus include tumour cells, virus-
infected cells and healthy but proliferating cells that
show increased levels of NPM/B23.

35   Preferred virus-infected cells are cells infected by the

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human immunodeficiency virus (HIV), for example CD4⁺ T-cells; or the human Papilloma virus (HPV), for example cervix epithelial cells and prostate epithelial cells; or a Rhinovirus, for example nasal epithelium cells.

Some preferred tumours include rapidly proliferating tumours in general; gliomas and other central nervous system tumours such as neuroblastomas; leukaemias; lymphomas; lung tumours; sarcomas; colon tumours such as carcinomas, e.g. low-grade colon tumours that have shown invasion (Duke III-IV); dispersed renal carcinomas; tubal carcinomas, gastric carcinomas; and prostate carcinomas.

Further, constructs of the invention may be used to target therapeutic agents to the nuclei of cells involved inflammatory conditions, as inflammatory conditions commonly involve cell proliferation. Examples of such inflammatory conditions include arthritis, particularly rheumatoid arthritis; arteritis; chondritis; cholitis; dermatitis; enteritis; myositis; tendosynobitis; and autoimmune inflammatory conditions such as SLE (systemic lupus erythematosis).

Fibroblasts are a further preferred cell type in which nuclear targeting may be achieved by constructs of the invention.

The cytostatic effect of protein H

As described in the Examples, protein H was found to have a cytostatic effect on murine B-lymphocytes, preventing them from proliferating but not inducing apoptosis (cell death). It is not yet clear whether protein H has this effect on all eukaryotic cell types. In situations where protein H and its fragments/derivatives are used to

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deliver other molecules to the nuclei of proliferating
cells, especially tumour cells, this cytostatic effect is
likely to be beneficial. In particular, in the case of
tumour cells, preventing the cells from proliferating
5   will complement the activity of the associated component
and thus assist in combatting the tumour.

In other situations, where it is not desirable to prevent
proliferation of the cells, it is preferred to use
10   fragments/derivatives of protein H that are capable of
being targeted to the nucleus but which do not exhibit
the cytostatic effect. A person of skill in the art will
be able to identify such fragments and derivatives by
preparing fragments/derivatives by means known in the art
15   and then testing them to determine whether or not they
(i) retain the ability to be targeted to the nucleus (see
"protein H" above); and (ii) exhibit the cytostatic
effect, which can be done using techniques based on those
given in the Examples e.g. by exposing the
20   fragments/derivative to proliferating cells and observing
whether or not they continue to proliferate.

Fragments and derivatives of protein H

25   For the purposes of the invention, a derivative of
protein H consists essentially of one of the four amino
acid sequences defined herein with respect to SEQ ID No.
6 (see sections entitled "protein H" and "sequence
information"). A fragment of protein H is a fragment of
30   any of these four sequences or a fragment of a derivative
of any of these four sequences. Such fragments and
derivatives are capable of being targeted to the nuclei
of eukaryotic cells as described above in the section
entitled "nuclear targeting".

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In particular, a derivative of protein H may be an
allelic variant or species homologue of protein H which
occurs naturally and is capable of being targeted to the
nucleus of a eukaryotic cell in a substantially similar
5   manner to the four versions of *Streptococcus pyogenes*
protein H as defined herein. Such allelic variants and
species homologues will typically be derived from other
bacteria, for example, other cocci such as species of
Staphylococcus or, preferably, *Streptococcus*.

10

Allelic variants and species homologues can be obtained
using techniques known in the art, based on the use of
probes derived from the *Streptococcus pyogenes* nucleic
acid coding sequence as probes. For example, such a probe
15   can be used to probe libraries made from bacterial cells
in order to obtain clones encoding the allelic or species
variants. The clones can be manipulated by conventional
techniques to express a protein which, according to the
invention, is a derivative of protein H.
20   Preferably, according to the invention, derivatives of
protein H have at least 70%, at least 80%, at least 90%,
at least 95%, at least 98% or at least 99% homology with
one of the four protein H sequences defined herein with
respect to SEQ ID No. 6. More preferably, a derivative
25   will have at least 95%, or at least 99% homology with one
of those four sequences over a region of at least 20,
preferably at least 30, for instance at least 40, 60 or
100 or more contiguous amino acids. Methods of measuring
protein homology are well known in the art and it will be
30   understood by those of skill in the art that in the
present context, homology is calculated on the basis of
amino acid identity (sometimes referred to as "hard
homology").

35   The sequence of derivatives of the invention may differ

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from that of one of the four protein H sequences defined
herein with respect to SEQ ID No. 6 by one or more amino
acid substitutions. For example, 1, 2, 3, 4, 5 to 10, 10
to 20 or 20 to 30 substitutions may be present, as long
as the derivative has the ability to be targeted to the
nucleus of a eukaryotic cell in a substantially similar
way to protein H. Preferably, substitutions are
conservative. For example, conservative substitutions may
be made according to the following table. Amino acids in
the same block in the second column and preferably in the
same line in the third column may be substituted for one
another in a conservative manner.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Similarly, derivatives of the invention may show one or
more deletions compared to any of the four protein H
sequences defined herein with reference to SEQ ID No. 6.
Each deletion may be deletion of, for example, 1, 2, 3, 4
or 5 to 10 amino acids.

Similarly, derivatives of the invention may show one or
more insertions compared to any one of the four protein H
sequences as defined herein with reference to SEQ ID No.
6. Each insertion may comprise, for example, 1, 2, 3, 4 5
to 10 or 10 to 20 amino acids.

For example, 1, 2, 3, 4 5 or more such deletions or

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insertions may be present.

According to the invention, fragments of protein H, and
of derivatives as defined above, may be of any length and
5   may be derived from any region of protein H or one of its
derivatives, as long as they have the capacity to be
targeted to the nucleus of a eukaryotic cell. For
example, suitable fragments may have a length of from 1
to 20 amino acids, from 20 to 50 amino acids, from 50 to
10   100 amino acids, from 100 to 150 amino acids, from 150 to
200 amino acids, from 200 to 250 amino acids, from 250 to
300 amino acids, from 300 to 350 amino acids, or greater
than 350 amino acids.

15   Preferably, fragments are derived from the more N-
terminal regions of protein H. This is because we have
found that the A region of protein H, and especially the
AB region (i.e. a fragment consisting of the A and B
regions) bind to NPM/B23 in a similar manner to complete
20   protein H. We have found that the AB region binds to
NPM/B23 more efficiently than complete protein H, and
that the A region also binds to protein H, albeit with
lower efficiency than complete protein H. The inhibition
experiments given in the Examples(see Figure 5C) also
25   suggest that the AB region may be capable of binding to
protein SET and hnRNP A2/B1.

This suggests that the A region, and preferably the AB
region, may be capable of being targeted to the nucleus.
30   Therefore, fragments of the invention preferably comprise
the A region, more preferably the AB region. Optionally,
other regions of the protein may also be present.

The AB region of protein H of Streptococcus pyogenes is
35   amino acids 1 to 117 in SEQ ID No. 6. Preferably, all of

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these 117 amino acids are present. However, a skilled
person will also be able to investigate smaller fragments
of the AB region to determine whether or not they retain
the capacity to be targeted to the nucleus. In
5   particular, recombinant techniques can be used to
generate such fragments and techniques based on those
given in the Examples can be used to determine whether or
not a given fragment is targeted to the nucleus.
Therefore, AB fragments may comprise, for example, 1 to
10   20, 20 to 50, 50 to 80, 80 to 100 or more of the 117
amino acids of the AB region. In such fragments, the AB
region may be truncated at its N-terminus by, for
example, 1 to 5, 5 to 10, 5 to 20 or 20 to 50 amino acids
and/or at its C-terminus by, for example, 1 to 10, 10 to
15   20 or 20 to 50 amino acids.

Optionally, derivatives of the invention may comprise
protein H or a fragment of protein H as defined herein,
and be extended at either the N or the C-terminus or both
20   by an unrelated amino acid sequence. For example, such a
sequence may be of up to 10, up to 20, up to 30, up to 50
or up to 100 amino acids in length, or longer (the
invention also provides fusion proteins comprising
protein H or a derivative or fragment thereof: see
25   below).

In the nuclear targeting constructs of the invention, a
revealing label may be present, and may be attached
either to protein H or the fragment/derivative of protein
30   H, or to one of the other components. The revealing label
may be any suitable label which allows the polypeptide to
be detected. Suitable labels include radioisotopes (e.g.
^{125}I, ^{35}S), an enzyme, an antibody, a polynucleotide or a
linker such as biotin.

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In the nuclear targeting constructs of the invention, the
protein H component, i.e. protein H or a fragment or
derivative thereof, may be present in a purely peptidyl
form. Alternatively, it may be chemically modified, e.g.
5   post-translationally modified. For example, it may be
glycosylated or comprise modified amino acid residues.

Fragments and derivatives of the invention may be
synthesised in any suitable manner. Typically, they will
10   be prepared by recombinant means. However, where
appropriate, they may also be made synthetically. For
example, they may be made using known techniques of
peptide synthesis. This applies especially to fragments
of protein H.

15

Another consideration is the IgG-binding properties of
protein H. In general, it is not desirable that
fragments/derivatives of protein H retain IgG-binding
properties. This is because binding to IgG may lead to
20   the formation of immune complexes which could lead to
undesirable side effects and/or reduce protein H's
capacity to exert a cytostatic effect. Thus, where the
construct of the invention is to be administered by a
route that allows the opportunity to form immune
25   complexes (notably intravenous injection), it is
preferred that the construct comprises a fragment or
derivative that has no capacity to bind to IgG, or at
least has a reduced capacity compared to intact protein
H. The IgG binding site is around 20 to 30 amino acids
30   long, and spans the boundary between the A and B regions.
Thus, it may be desirable to use a derivative of protein
H in which this region is deleted or mutated so that it
has no capacity, or a reduced capacity, to bind to IgG.
Of course, any such modifications should preferably be
35   made without appreciably disrupting the nuclear targeting

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effect of the invention. Thus, it is preferred that such derivatives retain the ability to interact with NPM, SET and hnRNPl.

5   It should also be noted that protein H naturally forms dimers. The dimers have a greater capacity to bind to IgG than isolated protein H monomers. Formation of dimers is favoured below 37°C (i.e. normal human body temperature)but the dimers are less stable above 37°C

10   (See, for example, Nilson et al Biochemistry, 1995, 34, pp13688-13698). Thus, the IgG binding capacity of protein H in vivo may actually be lower than in vitro experiments below 37°C suggest. For this reason, even complete protein H does not necessarily have a great enough IgG

15   binding capacity in vivo to disrupt the nuclear targeting effect of the invention.

Components which may be targeted to the nucleus when associated with protein H and its fragments/derivatives

20

In principle, the other component of the nuclear delivery construct may be of any nature, as long as its targeting to the nucleus is desired. For example, the other component may be a nucleic acid, i.e. polypeptide, i.e. a

25   DNA or RNA; a polypeptide, i.e. a protein or peptide, for example an antigen or antibody, e.g. an antibody to a nuclear protein; a small molecule drug; a liposome; or a detectable marker.

30   So far as nucleic acids are concerned, the nuclear targeting properties of protein H represent a novel means of carrying out gene therapy by delivering DNA or RNA to the nucleus. Thus, nucleic acids encoding a protein can be delivered to the nucleus, with a view to their being

expressed in the nucleus. For example, the nucleic acid
may be contained within a nucleic acid construct that
effects integration of the nucleic acid into the genome
of the cell. Alternatively, the nucleic acid may be
5   contained within a nucleic acid construct that is
designed to replicate within the cell without integration
into the genome of the cell, for example within a so-
called "mini-chromosome".

10   This avoids the well-known difficulties associated with
viral vectors, e.g the risk of uncontrolled viral
multiplication.

Protein H and its fragments/derivatives can be used to
15   target antisense nucleic acids to the nucleus, with a
view to their forming a duplex with RNA produced in the
nucleus and suppressing the expression of the protein it
encodes. The constructs of the invention may thus be
associated directly with antisense RNA, or they may be
20   associated with a construct encoding an antisense RNA,
which is transcribed in the nucleus of the cell.

Where the targeting constructs of the invention are used
to deliver antisense nucleic acids to cancer (tumour)
25   cells, antisense RNA can be used to suppress the
expression of proteins involved in the cancer phenotype.
For example, antisense RNA may be used to suppress the
expression of proteins from *myc*, *bcl2*, *bclx* or cyclin
genes.
30

Alternatively, antisense RNA to the RNA transcribed from
an HLA-DM gene may be used. Nucleophosmin appears to be a
common target in chronic graft-versus-host disease after
transplantation. Thus, possible modulation of NPM
35   expression by protein H or derivatives thereof in

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transplanted organs may be developed as a means of
reducing the incidence and severity of graft-versus-host
disease.

5   HLA-DM is involved in loading peptides on to MHC class II
        molecules, which leads to their presentation as antigens
        to CD4 T cells and, ultimately, to the recognition of
        foreign peptides by T cells. This phenomenon underlies
        graft-versus-host disease, as recognition of foreign
10   peptides by T cells is at least partly responsible for
        the rejection of grafted tissue. In the absence of HLA-
        DM, only the invariant clip peptide is presented, which
        does not provoke the host/graft reaction. Therefore,
        antisense RNA to HLA-DM may be useful in to interrupting
15   the process by which foreign peptides are presented as
        antigens by MHC class II molecules, preventing their
        recognition as antigens by T cells and thus preventing
        the rejection of grafted tissue by the host tissue and
        promoting its tolerisatiuon. This potentially applies
20   both to allografts (where the grafted tissue come from
        the same species as the recipient host) and to xenografts
        (where the grafted tissue comes from another species).

        So far as small molecule drugs are concerned, one
25   possibility is to use protein H and fragments/derivatives
        of protein H to deliver cytotoxic agents. Thus, if
        protein H exhibits selective nuclear targeting to cells
        having enhanced B23 expression, particularly tumour
        cells, protein H represents a way of targeting a
30   cytotoxic agent to a tumour cell, with a view to killing
        the tumour cell.

        Another possibility is to use protein H and
        fragments/derivatives of protein H to deliver peptides to
35   the nucleus. Some preferred peptides are those which

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chelate certain metal ions, for example $^{2+}$ ions, such as
Zn^{2+}, Mn^{2+} or Mg^{2+}. Many DNA processing enzymes and
transcription factors require $^{2+}$ ions and limiting the
supply of such ions by chelation could reduce the
5   division of cells to which the peptides are targeted.
This is expected to be useful in the treatment of
tumours, where chelation of certain ions may prevent the
proliferation of the tumour cells. For example, targeting
of chelating peptides that chelate a particular ion will
10   selectively inhibit certain DNA processing enzymes and
transcription factors. If desired, a cytotoxic effect can
also be achieved by increasing the amount of chelating
peptide used.

15   Other preferred peptides are those that interact with
transcriptional activators or co-activators involved in
regulating tumour cell growth or inflammatory responses,
the aim being, respectively, to reduce tumour cell growth
or to prevent or reduce the inflammatory response.
20

Where protein H or a fragment or derivative thereof is
attached to a detectable marker, any suitable marker
which allows the construct to be detected may be used.
Suitable markers include radioisotopes (e.g. ^{125}I, ^{35}S),an
25   enzyme, an antibody, a polynucleotide or a linker such as
biotin. In particular, markers that allow detection of
the construct by imaging techniques are preferred. Such
imaging techniques may be used in diagnostic methods.

30   Thus, if protein H is selectively targeted to the nuclei
of certain cell types, it could be used as a marker for
those cell types. In particular, if protein H is
selectively targeted to the nuclei of certain tumour
cells, or certain virally infected cells, then protein H
35   or fragments/derivatives thereof can be labelled and used

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as a marker for those cell types. This can be used to diagnose the presence of such cells. Thus, suitably labelled, protein H or fragments/derivatives thereof can be used to diagnose the presence of tumour or virally

5   infected cells to which protein H is selectively targeted. Once such imaging techniques reveal that protein H or a fragment or derivative thereof accumulates in the nuclei of the cells of a given tumour, or type of tumour, a further construct of the invention can also be

10   used to target therapeutic agents to that tumour, or type of tumour.

In the nuclear delivery constructs of the invention, there may be one component which is protein H or a

15   fragment or derivative thereof, as defined herein, and one component which is another molecule (i.e. a 1:1 ratio). Alternatively, the two components may be present with a different ratio, for example 1:2, 1:3, 1:4, 1:5 or higher; or 5:1 or greater, 4:1, 3:1, 2:1 or 1:1. Where

20   there is more than one component associated with protein H, these components may be the same or different. Similarly, there may be more than one molecule of protein H and more than one molecule of the other component, e.g a ratio of 2:2, 3:3, 2:3 or 3:2.

25

Association between protein H and its fragments/derivatives and the other component(s) of the nuclear delivery construct

30   The other component(s) may be associated with protein H in any manner, as long as the association is strong enough for protein H to transport the other component(s) into the nucleus. The other component(s) may therefore be associated with protein H by any means known in the art.

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For example, the association may be covalent associated.
Alternatively, the association may be non-covalent.

For example, the other component(s) may be conjugated to
5   protein H or a fragment or derivative of protein H. Where
the other component(s) is/are covalently attached, this
attachment may be at either the C-terminus or the N-
terminus, or anywhere between the two termini. In
constructs where the other component is a polypeptide, it
10   may be attached by a peptide bond.

Where protein H or a fragment/derivative is non-
covalently associated with the other component(s), the
association may be, for example, by means of hydrophobic
15   interactions, hydrogen bonding or electrostatic
interactions.

Where the other component is peptidyl in nature; e.g. a
protein, polypeptide or peptide, it may be desirable to
20   produce the construct as a fusion protein between protein
H and the second protein. Thus, a composite DNA sequence
encoding protein H or a fragment/derivative and the other
peptidyl component can be prepared by techniques known in
the art and a fusion protein can be expressed
25   recombinantly from this composite sequence. Optionally, a
linker nucleic acid sequence may be provided between the
two coding sequences, such that a linker region is
present in the expressed fusion protein. Alternatively,
the coding sequences of protein H or a
30   fragment/derivative of protein H may be joined directly
together in-frame. Fusion proteins are discussed in more
detail below.

Fusion proteins

35

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In a fusion protein of the invention, protein H and the other peptidyl component may be present in either orientation, i.e. protein H or a fragment or derivative thereof may be C-terminal or N-terminal to the other
5   peptidyl component. A linker peptide may be present between the two components. Typically, the linker will be flexible, allowing movement of the protein H component with respect to the other peptidyl component. Preferably, the linker will not inhibit the correct expression or
10   folding of either of the two components. Preferably, the linker will not be toxic or immunogenic.

Typically, the peptide linker comprises amino acids that do not have bulky side groups and therefore do not
15   obstruct the folding of the protein component. Further, it is preferred to use uncharged amino acids in the linker. Preferred amino acids for use in linkers include glycine, serine, alanine and threonine.

20   The peptide linker may be of any suitable length which allows correct folding of the two components. The linker may be from one to four amino acids in length. Alternatively, the linker may be from 5 to 50 amino acids in length, for example 10 to 30 amino acids or 15 to 25
25   amino acids in length.

Preferably the linker consists essentially of one or more glycine residues and one or more serine residues. Such a linker is termed herein a glycine-serine linker. The
30   linker may contain from 1 to 50, typically 5 to 30 or 10 to 20 glycine residues. The linker may contain from 1 to 50, typically 5 to 30, or from 10 to 20 serine residues.

The linker may consist only of glycine and serine
35   residues. One possible type of glycine-serine linker may

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comprise the sequence (Gly-Gly-Gly-Gly-Ser)$_n$. n may be
any integer from 2 to 10, preferably from 2 to 5, more
preferably 3 or 4, most preferably 3. Other combinations
of glycine and serine can also be suitable linkers.

The invention also provides nucleic acid sequences, i.e.
DNA and RNA sequences, encoding the fusion proteins of
the invention. The invention also provides: vectors
comprising these nucleic acid sequences; cells containing
such vectors or nucleic acid sequences; and methods of
producing fusion proteins of the invention, comprising
expressing the nucleic acid sequence encoding the fusion
protein in a cell, and recovering the fusion protein thus
obtained.

A person of skill in the art will be able to generate
nucleic acid sequences encoding fusion proteins by
techniques known in the art, and will also be able to
generate vectors comprising those sequences and transform
or transfect cells with such vectors in order to achieve
expression of the fusion protein.

Typically, in a vector, the nucleic acid sequence
encoding the fusion protein will be operably linked to a
control sequence capable of providing for the expression
of the fusion protein in the host cell. The term
"operably linked" refers to a juxtaposition wherein the
coding DNA sequence and the control sequence are in a
relationship which permits expression of the coding
sequence under the control of the control sequence.
Typically, the control sequence will be a promoter.

Optionally, other components may be present in the
vector, for example, any of the following: an origin of
replication; an enhancer; a selectable marker gene,

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typically under the control of a promoter; a terminator
sequence; or a polyadenylation sequence. Any such
components will be positioned such that they are operably
linked to the sequence encoding the fusion protein, i.e.
5   in a position such that they exert the desired effect on
the coding sequence.

Medical applications of the invention

10   Nuclear delivery constructs of the invention can be used
to deliver components as described above, in association
with protein H or a fragment/derivative thereof, to the
nucleus. This will be useful in combatting a number of
diseases of the human or animal body.
15

In particular, as NPM/B23 is up-regulated in tumour and
proliferating cells, nuclear delivery constructs of the
invention will be useful in combatting diseases of
proliferating cells, or diseases that involve
20   uncontrolled cell proliferation. In particular, nuclear
delivery constructs of the invention can be used to
deliver components to tumour cells, thereby combatting
the tumour.

25   In this connection, it is desirable to use nuclear
delivery constructs of the invention in which the
component associated with protein H or a
fragment/derivative thereof is a tumour-suppressive drug
or cytotoxic drug. As indicated above, the cytostatic
30   effect of protein H may complement the effect of these
drugs.

Some preferred tumours for such delivery include rapidly
proliferating tumours in general; gliomas and other
35   central nervous system tumours such as neuroblastomas;

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leukaemias; lymphomas; lung tumours; sarcomas; colon
tumours such as carcinomas, e.g. low-grade colon tumours
that have shown invasion (Duke III-IV); dispersed renal
carcinomas; tubal carcinomas, gastric carcinomas; and
prostate carcinomas.

Similarly, nuclear delivery constructs of the invention
can be used to target drugs to virus-infected cells, thus
combatting the viral infection. Preferred virus-infected
cells are cells infected by the human immunodeficiency
virus (HIV), for example CD4⁺ T-cells; or the human
Papilloma virus (HPV), for example cervix epithelial
cells and prostate epithelial cells; or a Rhinovirus, for
example nasal epithelium cells. Thus, where a nuclear
delivery construct of the invention comprises protein H
or a fragment or derivative thereof in association with
appropriate antiviral agent, the construct can combat
infection by one of these viruses.

Further, as inflammatory conditions often involve cell
proliferation, constructs of the invention may be used to
target therapeutic agents to the nuclei of cells involved
inflammatory conditions, thus treating these conditions
Examples of such inflammatory conditions include
arthritis, particularly rheumatoid arthritis; arteritis;
chondritis; cholitis; dermatitis; enteritis; myositis;
tendosynobitis; and autoimmune inflammatory conditions
such as SLE (systemic lupus erythematosis).

Also, where the nuclear delivery construct comprises DNA
or RNA, gene therapy can be effected. Any suitable gene
may be delivered to the nucleus in this way.

Where the targeting constructs of the invention are used
to deliver antisense nucleic acids to cancer (tumour)

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cells, antisense RNA can be used to suppress the
expression of proteins involved in the cancer phenotype.
For example, antisense RNA may be used to suppress the
expression of proteins from *myc*, *bcl2*, *bclx* or cyclin

5   genes, thus combatting the cancer concerned.
Alternatively, as described above, antisense RNA to the
RNA transcribed from an HLA-DM gene may be used, with a
view to combatting graft-versus-host disease.

10   The use of nuclear targeting constructs of the invention
in the treatment of a condition may be combined with
other treatments. In particular, where treatment of a
tumour is desired, it may be combined with or used in
association with other chemotherapeutic or

15   chemopreventive agents for providing therapy against
tumours. Similarly, it may be combined with the use of
other agents for treatment of viral infections or other
conditions.

20   Nuclear targeting constructs of the invention may be also
be used in diagnostic methods. For example, as described
above, protein H or a fragment or derivative thereof may
be associated with a detectable marker, especially an
imaging agent.

25

Pharmaceutical compositions

The present invention also provides pharmaceutical
compositions comprising a nuclear delivery construct

30   according to the invention and a pharmaceutically
acceptable carrier.

Any suitable pharmaceutical formulation may be used. For
example, suitable formulations may include aqueous or

35   non-aqueous sterile injection solutions, which may

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contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the bodily fluids of the intended recipient; and aqueous and non-aqueous sterile

5   suspensions which may include suspending agent and thickening agents. Some preferred formulation ingredients include mannitol or another sugar and/or phosphate-buffered saline (PBS).

10   It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question.

15

Dosage information

According to the invention, any effective, non-toxic amount of a nuclear deliver construct of the invention

20   may be administered to a patient. The dose of the nuclear delivery construct may be adjusted according to various parameters, for example the nature of the component which is being delivered to the nucleus, the age, weight and condition of the patient to be treated, the mode of

25   administration used, the condition to be treated, the efficiency of the particular nuclear delivery construct being used in transporting the associated component to the nucleus and the required clinical regimen.

30   As a guide, it appears that 10^6 B-lymphocytes accumulate in the region of 1ng to 10ng of protein H in their nuclei.

This suggests that the amount of nuclear delivery

35   construct administered may be such that the dose

comprises 10^{-14}g to 10^{-9}g of protein H or a fragment or
derivative thereof per cell to which the construct is to
be delivered, for example 10^{-14}g to 10^{-8}g, e.g. in the
region of 10^{-14}g, 10^{-13}g, 10^{-12}g, 10^{-11}g, 10^{-10}g, 10^{-9}g or 10^{-8}g
per cell. The amount of protein H or a fragment/
derivative thereof thus depends on what cells it is
desired to deliver the construct to, and how many of them
there are. The total amount of the construct delivered
also depends on the size of the component(s) associated
with protein H or the fragment/derivative.

For example, a typical dose of a nuclear delivery
construct of the invention might contain 1 to 1000μg of
protein H or a fragment/derivative thereof, for example 1
to 10μg, 10 to 100μg or 100 to 1000μg.

These dosages are intended only as a guide since a
skilled medical practitioner will be able to determine
readily the most appropriate dosage for any particular
patient and condition.

Similarly, the skilled medical practitioner will be able
to determine the appropriate dosage schedule, which will
vary according to the factors given above in respect of
dosage amounts. However, single doses and multiple doses
spread over periods of days, weeks or months are
envisaged.

Routes of administration

Nuclear delivery constructs of the invention can be
formulated for clinical administration by mixing them
with a pharmaceutically acceptable carrier or diluent, as
described above. For example, they can be formulated for

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topical, parenteral, intravenous, intramuscular or
transdermal administration. Of course, the route of
administration will be tailored to the particular
condition to be treated.

5

For example, where complete protein H, or a
fragment/derivative comprising the IgG binding site is
used in the construct, it may not be desirable to inject
the protein or fragment/derivative intravenously, as this
10   may lead to the formulation of immune complexes with IgG.
In these situations, other means of administration are
preferred, e.g. direct delivery of the construct to the
site where it is needed. For example, in the case of a
tumour, it is desirable to inject the protein or
15   fragment/derivative directly into the tumour. However, as
noted above, even the IgG binding capacity of complete
protein H is not necessarily enough to disrupt the
nuclear targeting effect of the invention.

20   **EXAMPLE**
The following Example illustrates the invention.

Summary

25   Some strains of the human pathogen *Streptococcus pyogenes*
express a surface protein called protein H, which is
released from the streptococcal surface by a cysteine
proteinase produced by the bacteria. Here we find that
soluble protein H binds to the surface of lymphocytes and
30   granulocytes. The molecule is taken up by lymphocytes and
transported to the nucleus through a previously unknown
intracellular pathway. In the cytoplasm, protein H was
found to bind to actin whereas when proteins were
solubilised from membrane fractions by papain, protein H

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was found to interact with nucleophosmin/B23, a protein
known to shuttle between the nucleus and the cytoplasm.
In the nucleus, protein H is dissociated from
nucleophosmin/B23 and instead forms complexes with the
nuclear proteins SET and hnRNP A2/B1, resulting in
nuclear accumulation of protein H and a cytostatic
effect.

Experimental procedures

**Bacterial strain, proteins, bacterial expression, *in
vitro* translation, labelling of proteins, coupling of
proteins to Sepharose**

The group A streptococcal strain AP1 of serotype M1
(Åkesson et al., 1994) was used. Recombinant protein H
and peptide fragments corresponding to the AB and A
regions of protein H have been described (Åkesson et al.,
1990; Frick et al., 1994). For the generation of *in vitro*
translated NPM, RNA was prepared from Jurkat T cells
using RNazol B (Tel-Test Inc., Friendswood, USA)
according to the manufacturer's recommendations. cDNA
synthesis was performed by incubation of 5 µg RNA with 1x
RT buffer (Gibco BRL), 1 mM dNTP, 10 mM DTT, 0.1 mg
BSA/ml, 4.5 µM poly dT_{18}, 20 U RNase inhibitor (Boehringer
Mannheim) and 200 U M-MLV reverse transcriptase (Gibco
BRL) in a final volume of 50 µl for 37°C for 1 hour. NPM
was PCR amplified using the 5' primer containing a NarI
site
5'-GCAGGGCGCCATGGAAGATTCGATGGACAT-3' (SEQ ID No. 1) and
the 3' reverse primer
5'-CAGGAATTCTTATTAAAGAGACTTCCTCCACTGCC-3' (SEQ ID No. 2)
containing an EcoRI site. For the generation of NPM
peptide fragments by *in vitro* translation two additional

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oligonucleotides were used for PCR amplification: The
NH2-terminal peptide was generated with the reverse
primer
5'-CAGGAATTCTTATTAGCTACCACCTCCAGGG-3' (SEQ ID No. 3)

5   and the primer
5'-TTGATGAAGGTTCCACAGAAAAAAGTAAAACTTGCTG-3' (SEQ ID
No.4), was used for the COOH-terminal peptide. The PCR
products were blunted and cut with EcoRI, whereas the
vector pGem-3Z was cut with HincII/EcoRI, and both were

10   ligated. *In vitro* translation was done using TNT Coupled
Reticulocyte Lysate Systems (SDS, Falkenberg, Sweden)
according to the manufacturer's recommendations.
Recombinant protein A was purchased from Pharmacia
Biotech, Uppsala, Sweden, and actin from porcine heart

15   was from Sigma (MO, USA). Protein H was labelled with ^{125}I
using the Bolton and Hunter reagent (Amersham, UK).
Protein fractions 85-87 purified with FPLC were
concentrated in an Amicon centricon concentrator (Amicon,
Inc., Beverly, MA), and labelled with ^{125}I using the

20   chloramine T method (Greenwood et al., 1963). ^{125}I was
from Nordion Int. Co. (Canada). Protein H was dialyzed
against 0.1 M NaHCO$_3$, pH 8.3 + 0.5 M NaCl, and coupled to
CNBr-activated Sepharose 4B (Pharmacia Biotech) as
previously described (Frick et al., 1995).

25

Electrophoresis and Western blot analysis

SDS-PAGE was performed as described by Laemmli (1970),
using a total polyacrylamide concentration of 10% or 13.6

30   % and 3.3% crosslinking. Samples were boiled for 3
minutes in a buffer containing 2% SDS and 5% 2-
mercaptoethanol. Gels were fixed with a mixture of 7%
acetic acid and 10% ethanol, dried and autoradiographed.
Molecular weight markers were from Sigma. Gels were

35   stained with Coomassie Blue. Protein fractions were

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applied to PVDF membranes (Immobilon, Millipore, Bedford,
MA, USA) using a Milliblot-D system (Millipore).
Membranes were blocked at room temperature for 1 hour in
VBS (10mM veronal, 0.15 M NaCl pH 7.4) containing 0.25%
5   Tween-20 and 0.25% gelatin. After incubation at room
temperature for 3 h with radiolabelled protein in VBS
containing 0.1% gelatin, the membranes were washed four
times with 1.0 M NaCl, 10 mM EDTA, pH 7.7, 0.25% Tween-20
and 0.25% gelatin. The filters were air-dried and
10   autoradiographed at -70°C using Kodak X-Omat AR films and
Kodak X-Omat regular intensifying screens.

**Cells and preparation of proteins from membrane fractions
from the Jurkat cell line**

15

For flow cytometric analysis human peripheral blood
lymphocytes (PBL) from healthy volunteers were depleted
from erythrocytes and prepared by Ficoll separation
(Pharmacia Biotech). Jurkat cells, a human T cell line,
20   were cultured in RPMI 1640 supplemented with 7.5% FCS and
20mM sodium pyruvate. Membrane preparations were
performed in the cold (0-4°C). 10^9 Jurkat cells were
homogenized in homogenization buffer (0.05 M Tris-HCl pH
7.5, 0.25 M sucrose, 0.005 M $MgCl_2$, 0.025 M KCl) followed
25   by centrifugation at 1000 x g for 10 min. The supernatant
was further centrifuged at 105000 x g for 45 min. This
supernatant was saved and used as a cytoplasmic fraction
whereas the pellet obtained was solubilised in 4 ml 0.01
M Tris-HCl pH 8.0. The protein content was determined
30   with the Coomassie protein assay reagent (Pierce, Boule
Diagnostics AB, Huddinge, Sweden). After addition of 0.2
mg papain (Sigma) per mg protein solution, and L-cysteine
(Sigma) to a final concentration of 4 mM, the mixture was
incubated at 37°C for 45 minutes. To terminate the
35   reaction, 4 ml ice-cold 0.01 M Tris-HCl pH 8.0 and

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iodacetamide (Sigma) to a final concentration of 6 mM
were added, followed by centrifugation for 1 hour at
105000 x g. Papain was removed from the supernatant by
chromatography on DEAE Sephadex A-50 (Pharmacia Biotech)
5   equilibrated with 20 mM Tris-HCl, pH 8.0. The column was
washed with 5 volumes 20 mM Tris-HCl pH 8.0, and the
material was eluted with 3 volumes of a 0.5 M NaCl in
this buffer followed by dialysis against 20 mM Tris-HCl
pH 8.0. For fractionation of the papain digested membrane
10   proteins, the solution was loaded onto an ion-exchange
column (Mono-Q, Pharmacia Biotech) mounted on a fluid
pressure liquid chromatograph (FPLC, Pharmacia Biotech).
Elution was performed with a 70 ml linear salt gradient
(from 0 to 1 M NaCl in 20 mM Tris-HCl pH 8.0). Fractions
15   of 0.5 ml were collected. Murine splenic B cells from
Balb/c mice were isolated as previously described
(Axcrona et al., 1995). Lymphocytes at 3×10^5/ml were
plated out in 96 well plates. B cells were activated with
LPS (25µg/ml, Difco) and incubated with proteins H and A
20   at indicated concentrations. One µCi [3H]thymidine was
added per well for the last 4 hours of a 40 hour culture
period, cells were harvested and processed for
scintillation counting. Values are mean +/- SD of
duplicates. For preparation of nuclear and cytoplasmic
25   extracts from murine B cells, cells were activated for 24
hours at 3.8×10^6 cells/ml with LPS (25µg/ml) and protein
H at 100µg/ml.

Preparation of nuclear extracts from Jurkat T cells and
30   **murine B cells**

Nuclei from Jurkat cells were isolated as described by
(Mirkovitch et al., 1984). Preparations of nuclei were
resuspended in 100 µl of buffer A (50 mM Hepes buffer, 50
35   mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10% glycerol)

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and additional buffer A was added to a final volume of
162 µl. 13 µl of 4 M (NH$_4$)$_2$SO$_4$ was added to bring the
final concentration to 0.3 M. The cells were rocked for
30 minutes and the viscous material was transferred to a
0.2 ml TLA-100 tube (Beckmann), followed by
centrifugation at 10^5 rpm for 10 minutes. 125 µl of the
supernatant was transferred to a second TLA-100 tube, 75
µl of 4 M (NH$_4$)$_2$SO$_4$ was added to increase the final
concentration to 1.5 M. The solution was centrifuged at
50000 rpm for 5 minutes. The supernatant was removed and
the pellet was resuspended in 100 µl of buffer A.
Extracts were used immediately or stored at -80°C.

**Affinity chromatography, competitive binding assay, and
plasmon resonance spectroscopy**

The ^{125}I-labelled pooled fractions 85-87 from Mono-Q and
in vitro translated NPM peptides were applied onto a
Protein A-Sepharose column (Pharmacia Biotech) and the
flowthrough fractions were run on a protein H-Sepharose
column. The column was extensively rinsed with PBSAT
(PBSA + 0.05% Tween-20). Bound proteins were eluted with
3 M KSCN and the radioactivity of the fractions was
measured in a gamma counter. Fractions were also analysed
by SDS-PAGE. Competitive binding assays were performed as
reported (Åkerström and Björck, 1989). Binding kinetics
were determined by surface plasmon resonance spectroscopy
using a BIACORE X system (Biacore AB, Uppsala, Sweden).
Actin and nuclear extracts purified on protein H-
Sepharose were immobilized on research grade CM5 sensor
chips in 10 mM sodium acetate at pH 4.0 and 4.5,
respectively, using the amine coupling kit supplied by
the manufacturer, whereas biotinylated NPM was coupled to
CM5 sensor chips precoupled with avidin (Biacore). All
measurements were carried out in PBST. Analyses were

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performed at 25°C and at a flow rate of 10 µl/min. To
calculate dissociation and affinity constants, 35 µl of
protein H or proteins A, H or actin were applied in
serial dilutions (2n; starting at 600 µg/ml). Surfaces
5   were regenerated with 35 µl 1 M KSCN at a flow rate of 10
µl/min. The kinetic data were analysed by the
BIAevaluation 2.2 program (Biacore).

Amino acid sequence analysis

10

Proteins were separated by SDS-PAGE and stained with
Coomassie Blue. Protein bands were excised and digested
in-matrix using trypsin. Peptide fragments were separated
by reverse-phase HPLC (Vydac 218TP, I.D. 1.6 x 250 mm)
15   and aliquots were analysed by automated Edman degradation
using a model 477A sequenator connected to a model 120A
on-line PTH-analyser (Applied Biosystems, Weiterstadt,
Germany) and by mass analysis using cyano-4-
hydroxycinnamic acid as a matrix on a Voyager-DE MALDI-
20   TOF mass spectrometer (Perseptive Biosystems, Wiesbaden,
Germany) (Herrmann et al., 1996; Herwald et al., 1996).
The Blast network server at the National Center for
Biotechnology Information (Altschul et al., 1990) was
used for sequence homology searching.

25

**Antibodies, flow cytometrical and fluorescence
microscopical analysis**

For flow cytometric analysis of human PBL, mouse anti-
30   human CD3, CD4, HLA-DP/DQ/DR (Becton Dickinson, San Jose,
CA, USA) and CD8 antibodies (Dako Patts, Gentofte,
Denmark) were used. As a positive and negative control
for Jurkat T cells, an isotype control set of a γ1-FITC,
γ2-PE and anti-CD45 PerCP (Becton Dickinson) labelled

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antibodies were used, where the γ1/γ2 antibodies were
unspecific fluorochrome-conjugated antibodies. Proteins H
and A were biotinylated as previously described (Axcrona
et al., 1995), and used in conjunction with FITC-coupled
5   avidin (Sigma). Mouse anti-human HLA-DP/DQ/DR antibodies
were detected with goat anti-mouse FITC-coupled antibody
(Becton Dickinson). FACS-analysis was performed on a
Becton Dickinson FACSort flow cytometer (Becton
Dickinson). Each dot blot and histogram represents the
10   analysis of 10^4 gated cells. For fluorescence microscopy
0.5×10^6 Jurkat cells were incubated with 20µg
biotinylated proteins in culture medium for 30 minutes on
ice in a flat bottomed 96 well plate. Cells were washed
once, followed by continued incubation in a culture
15   chamber at 37°C for the indicated times. The cells were
washed twice in 4 ml PBS, taken up in 400 µl PBS,
centrifuged on slides in a cytocentrifuge (Shandon,
Cytospin 2) at 550 RPM for 3 minutes. After incubation
with FITC-coupled Avidin (Sigma), the cells were examined
20   in a fluorescence microscope (Leica Aristoplan) and
photographed.

Experimental procedures using anti-protein H or anti-NPM antibodies

25

Preparation of anti-protein H F(ab')$_2$ fragments
Anti-protein H antiserum was applied to a protein G-
Sepharose column. The column was extensively washed with
PBS and bound IgG was eluted with 0.1 M glycin-HCl pH
30   2.0. Eluted IgG was dialyzed against acetate buffer pH
4.5 (70 mM CH_3COONa - 50 mM HCl) followed by proteolytic
cleavage with pepsin (ratio of protein:pepsin was 100:1)
for 21 hours at 37°C. The reaction was terminated by
raising the pH of the solution to 7.5 with 1 M Tris and

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uncleaved IgG was removed by subjecting the material to affinity chromatography using protein G-Sepharose. Unbound material corresponding to polyclonal anti-protein H F(ab')₂ fragments was collected and dialyzed against

5   PBS. Coupling of F(ab')₂ fragments to Sepharose 4B (Pharmacia Biotech) was performed as recommended by the manufacturer.

Cloning and expression of nucleophosmin (NPM)

10   For expression in *E. coli* NPM was PCR amplified using the 5' primer containing an EcoRI site 5'-GCAG_GAATTC_ATGGAAGATTCGATGGACAT-3' (SEQ ID No 7) and the 3' reverse primer 5'-ATA_GCGGCCGC_TTATTAAAGAGACTTCCTC-3' (SEQ ID No 8) containing a NotI site. The DNA was cloned

15   into the prokaryotic expression vector pGEX-6p-1 (Pharmacia Biotech) using the EcoRI and NotI sites. Recombinant NPM fused to Glutathione S-transferase (GST) was expressed and purified according to the manufacturers instructions. After purification on Glutathione

20   Sepharose, the GST tag was cleaved off using PreScission[a] Protease (Pharmacia Biotech). From 1 l of an over night culture, approximately 1 mg pure NPM was achieved. Antibodies against NPM were raised in rabbits.

25   **Preparation of cytoplasmic and nuclear extracts from Jurkat T cells and Detroit cells**

Jurkat T cells and Detroit 562 human (carcinoma) pharynx epithelial cells (ATCC CCL 138), incubated with various proteins, were washed five times with PBS, resuspended in

30   buffer A (10 mM Hepes, 15 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 1mM PMSF, 1 µg antipain/ml , 0.5 µg leupeptin/ml) and the cell membranes were lysed using 0.2% NP-40. The cytoplasmic fractions were collected after centrifugation at 1000 x g for 10 min and the

-40-

nuclear pellets were washed twice with PBS and
resuspended in 100 µl of buffer B (50 mM Hepes buffer, 50
mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10% glycerol).
Additional buffer B was added to a final volume of 162 µl

5   and 13 µl of 4 M $(NH_4)_2SO_4$ was added to bring the final
concentration to 0.3 M. The resuspended nuclei were
rocked for 30 min at 4°C and the viscous material was
transferred to a 0.2 ml TLA-100 tube (Beckmann), followed
by centrifugation at 350 000 x g for 10 min. The

10   supernatants corresponding to the nuclear fractions were
collected and both cytoplasmic and nuclear fractions were
subjected to immunoprecipitation.

15   **Immunoprecipitation**
Extracts prepared from Jurkat cells, incubated with
protein H or protein L (150 µg) for 16 hours at 37°C,
were immunoprecipitated by using polyclonal antibodies
against protein H or protein L, 2 µl respectively, for 2

20   hours at 4°C. 40 µl protein A-Sepharose was added and
incubation was continued for 16 hours at 4°C. Extracts
from Detroit 562 cells, incubated with protein H were
immunoprecipitated similarly.
Alternatively extracts from Jurkat cells, incubated with

25   protein H (150 µg) for various timepoints at 37°C, were
precleared with 50 µl glycine-Sepharose, for 2 hours at
4°C, followed by immunoprecipitation using polyclonal
anti-protein H F(ab')2-Sepharose (100 µl) for 16 hours at
4°C.

30   The Sepharose pellets were then washed three times with
PBS, boiled for 3 min in buffer containing 2% SDS and 5%
2-mercaptoethanol followed by centrifugation at 8000 x g
for 5 min. Supernatants were recovered and subjected to
SDS-PAGE and Western blot analysis. Membranes were

35   blocked at 37°C in PBST (PBS + 0.05% Tween-20) containing

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5% skim milk and probed with polyclonal antibodies
followed by peroxidase-conjugated protein A and developed
with ECL.

5   Results

        Protein H interacts with human lymphocytes and
        granulocytes

10   Protein H is released from the streptococcal surface
        through the action of a cysteine proteinase produced by
        the bacteria (Berge and Björck, 1995). The *E. coli*-
        produced fragment of protein H used in this study is
        similar in size to the fragment released by the
15   streptococcal enzyme, and in the following Examples
        protein H refers to this COOH-terminally truncated
        fragment expressed by and purified from *E. coli*. The
        interaction of protein H with the surface of T cells and
        granulocytes was analysed with flow cytometry. Human
20   peripheral blood lymphocytes were incubated with protein
        H and the majority (>90%) of the CD3^{+}, CD4^{+} and CD8^{+} cells
        bound protein H (Figure 1A). When the binding of protein
        H to cells within the granulocyte gate was analysed,
        protein H also stained these cells brightly (Figure 1A).
25   Protein H binds to IgGFc (Frick et al., 1994) and
        previous work has indicated affinity also for human MHCII
        antigens (Åkesson et al., 1994). The human Jurkat T cell
        line was therefore chosen for the subsequent experiments
        as MHC II expression on these cells could be excluded
30   with flow cytometry. More than 97% of the Jurkat T cells
        were found to be protein H^{+} as compared to the FITC
        avidin background. Furthermore, as shown in Figure 1B,
        Jurkat cells were stained by anti-CD45 but not with
        unspecific γ1 FITC/γ2 PE mouse mAbs.

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**Protein H is taken up by lymphocytes and accumulated in
the nucleus**

5   Biotinylated protein H was incubated with Jurkat cells
     for different timepoints. Following incubation, cells
     were cytospinned, fixed, and FITC-coupled avidin was
     added. As demonstrated by immunofluorescence microscopy
     (Figure 2) protein H was gradually accumulated in the
10   nuclei, and after 8 hours 80% of the nuclei showed
     staining. In contrast, cytoplasmic staining but no
     labelling of nuclei was detected when the Jurkat cells
     were incubated with biotinylated protein A (Figure 2C).
     Like protein H, protein A of *Staphylococcus aureus* is an
15   IgGFc-binding bacterial surface molecule. Previous work
     has demonstrated that protein H binds to murine and human
     B cells (Axcrona et al., 1995) and as in the case of
     Jurkat cells, protein H was targeted to the nuclei of the
     human B cell line Bjab, whereas protein A showed no
20   nuclear accumulation.

     Protein H are taken up by lmphocytes and epithelial cells
     Further studies were carried out using anti protein H
     antibodies. 5 x 10^{6} Jurkat cells were separately
25   incubated with 150 µg of protein H or protein L for 16
     hours. Cytoplasmic and nuclear extracts were prepared and
     immunoprecipitation was performed using polyclonal
     antibodies against proteins H and L, respectively,
     followed by the addition of protein A-Sepharose.
30   Precipitated materials were run on SDS-PAGE and blotted
     to PVDF membranes. The membranes were probed with
     polyclonal antibodies against proteins H and L,
     respectively, followed by peroxidase-conjugated protein A
     and developed with ECL. Protein H was taken up by the

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cells and could be detected in both cytoplasmic and
nuclear extracts, whereas protein L was not taken up by
the cells (not shown).
Epithelial cells (Detroit 562) were also incubated with

5   protein H (150 µg) and extracts prepared from these cells
were immunoprecipitated as described above. Protein H
was taken up by the Detroit cells, although a lower
amount of protein was detected in the nuclear extracts.

10   **Protein H interacts with nucleophosmin/B23 and actin**

To identify proteins interacting with protein H and
mediating its uptake, membrane preparations obtained by
subcellular fractionation of Jurkat cells were treated

15   with detergent (NP-40). However, no protein H-binding
proteins could be detected in this solubilised material
before or following purification by ion-exchange
chromatography, gel filtration or affinity chromatography
on protein H-Sepharose. To release water soluble peptides

20   from Jurkat cell membrane preparations, papain was used.
The solubilised peptides were separated by ion-exchange
chromatography and fractions were eluted by a linear
sodium chloride gradient (Figure 3A). The fractions were
applied in slots to PVDF membranes and probed with

25   radiolabelled protein H. Fractions 85-87 reacted with the
probe, and they were pooled. A portion (20 ml) was
labelled with ^{125}I and subjected to affinity
chromatography on protein A-Sepharose (Figure 3B). The
labelled peptides showed no affinity for protein A but

30   when the run-through fractions from protein A-Sepharose
were applied to protein H-Sepharose more than 70% of the
radioactivity was bound and eluted with 3M KSCN (Figure
3B). When analysed by SDS-PAGE and autoradiography, this
material (peak II) contained two major bands of 18 and 54

35   kDa, respectively. The run-through material (peak I) gave

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rise to a single band with a molecular mass of
approximately 16 kDa. Unlabeled pool 85-87 material was
now purified on protein H-Sepharose followed by SDS-PAGE.
After staining, the 18 and 54 kDa bands (see Figure 3C)
5   were cut out of the gel, digested with trypsin and
separated by HPLC. The amino acid sequences shown in
Figure 3C could be determined from HPLC peaks and
demonstrated that both bands contained nucleophosmin/B23
(NPM), a protein known to shuttle between the cytoplasm
10   and the nucleus (Borer et al., 1989). Monomeric NPM has a
molecular mass of 32 kDa but the protein is known to form
oligomers (Schmidt-Zachmann et al., 1987; Herrera et al.,
1996), including dimers of 70 kDa also under denaturing
conditions (see Umekawa et al., 1993 and Figure 3D).
15   Therefore the 54 kDa band probably consists of dimers of
NPM fragments generated by papain cleavage whereas the 18
kDa fragments do not form multimers in SDS-PAGE.
Intact NPM and two fragments of NPM covering the NH_2- and
COOH-terminal halves of NPM, respectively, were generated
20   by PCR and in vitro translation. These ^{35}S-methionine-
labelled peptides were separated by SDS-PAGE followed by
autoradiography (Figure 3D, left). As mentioned above,
NPM has a tendency to form dimers-oligomers (Schmidt-
Zachmann et al., 1987; Umekawa et al., 1993; Herrera et
25   al., 1996). This property is evident for the intact
molecule giving rise to bands of 35 and 70 kDa
corresponding to monomers and dimers. In case of the NH_2-
terminal fragment with an apparent molecular mass of 18
kDa, a band corresponding to a trimer of 54 kDa is seen,
30   whereas no distinct oligomers are present in lane 3 where
the COOH-terminal fragment was run. This is consistent
with previous observations demonstrating that the COOH-
terminal part of NPM is not essential for oligomerization
(Herrera et al., 1996). When the three NPM peptides were
35   subjected to affinity purification on protein H-

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Sepharose, intact NPM and the NH$_2$-terminal fragment bound
to protein H. The COOH-terminal fragment, however, did
not show affinity for protein H-Sepharose (Figure 3D,
right). These results and the fact that the 18 kDa NH$_2$-
5   terminal papain fragment of NPM shown in Figure 3C, also
has affinity for protein H, map the binding of protein H
to the NH$_2$-terminal part of NPM. As the COOH-terminal
region of NPM contains the signals essential for its
localization to the nucleolus (Wang et al., 1993),
10   binding of protein H should not interfere with the
targeting of NPM.

Using fragments of protein H in competitive binding
experiments the region of protein H interacting with NPM
15   was identified. Protein H was immobilized on Sepharose
and radiolabelled NPM (pool 85-87) was added. 80-90
percent of the radioactivity was bound to the Sepharose.
As demonstrated in Figure 3E this binding was inhibited
by unlabeled protein H and by fragments A and AB of
20   protein H, whereas the effect of protein A was at
background level also at high concentration. The
inhibition with the NH2-terminal fragment AB, which is an
even more efficient inhibitor than intact protein H, maps
the binding of NPM to this region.

25

The membrane material subjected to papain digestion was
obtained by a two-step centrifugation procedure where the
supernatant following the final centrifugation at 105000
g represents a cytoplasmic fraction. Also this material
30   was subjected to affinity chromatography on protein H-
Sepharose. A dominating band with an apparent molecular
mass of 40 kDa was eluted, and NH$_2$-terminal amino acid
sequencing established that the band was actin.

35   **Nucleophosmin is co-precipitated with protein H.**

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1 x 10^{6} Jurkat cells were incubated with protein H (150
µg) for various timepoints and in order to analyse if
nucleophosmin could be coprecipitated with protein H,
nuclear extracts were prepared. The extracts were
5   precleared with glycine-Sepharose and immunoprecipitated
using anti-protein H F(ab')$_2$-Sepharose. Precipitated
materials were run on SDS-PAGE and blotted to a PVDF
membrane. The membrane was probed with polyclonal
antibodies against recombinant nucleophosmin, followed by
10   peroxidase-conjugated protein A and developed with ECL.
Nucleophosmin could be detected in the nuclear extracts.
Thus, nucleophosmin could be coprecipitated using
antibodies against protein H demonstrating an *in vivo*
interaction between nucleophosmin and protein H.

15   **Identification of nuclear proteins interacting with
protein H**

Unlike the homogeneous nuclear staining seen with protein
H, anti-NPM antibodies detect a granular accumulation of
20   NPM in nucleoli (Borer et al., 1989). To investigate
whether protein H after the entry into the nucleus,
presumably together with NPM, interacts with nuclear
proteins, nuclear extracts from Jurkat cells were run on
protein H-Sepharose. Several bands were eluted (see
25   Figure 4, STAIN, lane 2) but when probed with
radiolabelled protein H, only three reacted with the
probe (Figure 4, BLOT, lane 2). These bands have apparent
molecular masses of 39, 42 and 80 kDa, respectively. The
39 and 80 kDa bands (I and III, respectively) were
30   identified as the SET protein by microsequencing. This
protein was initially described as an oncogene product
fused to a protein called CAN (v. Lindern et al., 1992).
Three tryptic fragments each of the 39 and 80 kDa bands
were subjected to NH$_2$-terminal sequencing and all
35   sequences were related to the SET protein (Figure 4,

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lower section). This fact and the molecular mass of the
80 kDa band, suggest that it represents a SET dimer. The
amino acid sequence of the 42 kDa band (band II, Figure
4) identified this band to be heterogeneous nuclear
5   ribonuclear protein (hnRNP) A2/B1, a member of the hnRNP
family (Dreyfuss et al., 1993).

Further analyses and comparison between the interactions
of protein H with actin, NPM and the nuclear proteins

10

To analyse the interactions between protein H and the
various intracellular proteins in more detail, plasmon
resonance spectroscopy was utilized. In these experiments
different amounts of protein H were applied and left to
15   interact with immobilized actin, NPM or with immobilized
protein H-binding nuclear proteins, to the level of
saturation (Figure 5A). The NPM used in these experiments
corresponds to the material shown in Figure 3C, and to
create an experimental situation similar to *in vivo*
20   conditions, a mixture of the nuclear proteins interacting
with protein H was used for both plasmon spectroscopy and
competitive binding experiments (see Figure 4, STAIN,
lane 2).

25   Figure 5A shows typical sensorgrams for the interactions
between protein H-actin, protein H-NPM and protein H-
nuclear proteins. On the basis of these experiments,
dissociation and association rates were calculated, and
used to determine association and dissociation constants
30   (Figure 5B). The data demonstrate that protein H has high
affinity for actin but also readily dissociates from the
complex, and that protein H has a higher association rate
and a considerably slower dissociation rate for the
nuclear proteins as compared to NPM. Also, competitive
35   binding experiments in which NPM and the nuclear proteins

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simultaneously compete for the binding of protein H,
showed that unlabeled nuclear proteins more efficiently
inhibited the interaction between radiolabelled NPM and
protein H-Sepharose, than unlabeled NPM itself . In
5   contrast, actin did not interfere with NPM-protein H
binding (Figure 5C) and neither did actin interact with
immobilized NPM (Figure 5A, middle section). In none of
these experiments did staphylococcal protein A show
affinity for actin, NPM or the nuclear proteins (Figure 5
10   A and C). In summary, the data on the binding kinetics
provide an explanation for the release of protein H from
its complex with NPM, and its nuclear accumulation as a
result of the binding to the nuclear proteins SET and
hnRNP A2/B1.

15

**Soluble protein H has a cytostatic effect on murine B
cells**

The interaction with the SET protein, a putative
20   transcription factor and oncogene product, and hnRNP
A2/B1, a molecule participating in mRNA processing,
suggested that protein H could interfere with various
cell functions and that metabolically active cells could
be particularly sensitive to protein H. It has been shown
25   that protein H interacts also with murine lymphocytes
(Axcrona et al., 1995), and the effect of protein H on
the proliferation of these cells was therefore
investigated. Initially we investigated whether protein H
when added to LPS-stimulated murine B cells, was
30   transported to the nucleus, and Western blot experiments
demonstrated that this was the case (Figure 6A). There is
no indication that protein H is degraded on its way from
the exterior of the cell into the nucleus. Thus, the
molecular mass of protein H identified in the medium, the

cytoplasm and the nucleus is very similar if not
identical. As a control, an identical PVDF membrane as in
Figure 6A was incubated with pre-immune serum followed by
peroxidase-conjugated protein A. No signals were
5   obtained. Following 24 hours of incubation with protein
H, the cytoplasm contained 1.4, and the nuclei 2.4ng of
protein H per 10^6 B cells. Moreover, addition of protein
H to the LPS-stimulated B cells, inhibited proliferation
measured as [^{3}H]Tdr-uptake in a dose dependent manner
10   (Figure 6B). A 50 percent inhibition was recorded at the
highest protein H concentration tested (50 µg/ml). As
determined by morphology and DNA laddering, protein H did
not induce apoptosis in the LPS-stimulated B cells.

15   **Legends to figures**

**Figure 1. Binding of protein H to the surface of human
peripheral blood lymphocytes and the human Jurkat T cell
line determined by FACS analysis.**

20

(A) Staining of T cells with CD3, CD4, and CD8 versus
protein H. Granulocytes were gated out with side
scatter/forward scatter and stained with protein H.
(B) Jurkat T cells were stained with protein H and CD45.
25   Background staining is shown with FITC avidin and CD3 on
the granulocyte gated cells and with g1-FITC/g2-PE
antibodies on Jurkat cells.

**Figure 2. Uptake and nuclear accumulation of protein H in
30   Jurkat T cells.**

Jurkat T cells were incubated with biotinylated proteins
H or A, cytospinned and stained with FITC coupled avidin.
(A) Incubation with protein H for four hours and (B) for

-50-

eight hours. (C) Incubation with protein A for eight
hours.

Figure 3. Protein H interacts with nucleophosmin.

5

(A) Membrane preparations from Jurkat T cells were
digested with papain and the solubilised peptides were
subjected to FPLC on a Mono-Q column. Fractions (0.5 ml)
eluted with a linear NaCl gradient were analysed for

10   protein H-binding activity in a slot binding assay, and
fractions 85-87 reacted with radiolabelled protein H. (B)
Radiolabelled pool 85-87 material was run on protein A-
Sepharose without showing affinity (left). In the right
section the pooled fractions of the run-through peak from

15   protein A-Sepharose were subjected to a protein H-
Sepharose column. (C) Peak fractions of peaks I and II
from protein H-Sepharose were separated by SDS-PAGE (10%
gel), followed by autoradiography. Unlabeled pool 85-87
material was run in parallel. This gel was stained and

20   bands marked by the arrows were cut out. The peptides of
the bands were digested with trypsin and tryptic
fragments were separated by HPLC. NH_2-terminal sequences
could be determined from one HPLC peak each of the two
bands. These sequences established that both bands

25   contained NPM. Numbers indicate amino acid residue
positions in the NPM sequences.(D) *In vitro* translated
and ^{35}S-methionine-labelled NPM peptides were separated by
SDS-PAGE (13.6% gel). The gel was dried and subjected to
autoradiography (left). Lane number corresponds to the

30   peptide run and the peptides are shown schematically in
the lower part of the figure. In the COOH-terminal
peptide 3, X indicates putative nuclear localization
signals. The three radiolabelled peptides were separately
applied to protein H-Sepharose. Following extensive

35   washing, bound material was eluted with 3M KSCN, dialyzed

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against PBS, concentrated and run on SDS-PAGE. The gel
was dried and autoradiographed (right). (E) Mapping of
the NPM-binding region of protein H by competitive
inhibition. The binding of ^{125}I-labelled NPM to protein H
5   immobilized on Sepharose was inhibited with different
amounts of unlabeled intact protein H, fragments AB and A
of protein H, or protein A.

Figure 4. Identification of nuclear proteins interacting
10   **with protein H.**

A nuclear extract was prepared from Jurkat T cells (lane
1). The extract was precleared with glycine-Sepharose
followed by incubation with protein H-Sepharose. After
15   extensive washing proteins bound to the protein H-
Sepharose were eluted with 3 M KSCN, dialyzed against PBS
and separated by SDS-PAGE (lane 2). Two identical gels
(10%) were run simultaneously; one was stained with
Coomassie blue (STAIN), one was blotted onto a PVDF
20   membrane and probed with ^{125}I-labelled protein H (BLOT).
Material corresponding to the three bands indicated was
submitted to trypsin digestion, HPLC and NH$_2$-terminal
sequencing. Three sequences were obtained from each of
bands I and III, showing identity to the SET protein.
25   Band II gave rise to two sequences found in hnRNP A2/B1.
The sequences are shown in the lower part of the figure,
and numbers indicate where homologous residues in the SET
and hnRNP A2/B1 are found.

30   **Figure 5. Analysis of the binding of protein H to**
nucleophosmin and nuclear proteins.

(A) Overlay plots of the binding of proteins H and A to
immobilized actin (left), NPM (middle) or nuclear

-52-

proteins (right) using plasmon resonance spectroscopy.
Increasing concentrations of protein H were applied for 3
min. each during association phase. Dissociation of bound
proteins was measured (expressed in resonance units, RU)
5   following injection of buffer alone.

Affinity rates and dissociation constants for the
interactions between protein H and actin, immobilized NPM
or nuclear proteins are as follows (values are mean ±
10   standard deviation from three experiments).

	Actin–Protein H	NPM–Protein H	Nuclear proteins–Protein H
Association rate $(10^3 \times s^{-1}M^{-1})$	2.5±0.1	6.7±0.82	1.7±0.5
Dissociation rate $(10^5 \times s^{-1})$	150±6.6	190±2.9	6.7±0.6
Association constant $(10^6 \times M^{-1})$	1.7±0.2	3.6±0.5	26.2±9.2
Dissociation constant $(10^{-8} \times M)$	60.0±5.7	28.6±3.7	4.4±1.7

25

(B) Competitive inhibition of the binding of ^{125}I-labelled
NPM to protein H-Sepharose with different amounts of
unlabeled NPM, nuclear proteins, protein A or actin.

30   **Figure 6. Nuclear uptake and cytostatic effect of protein
        H.**

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(A) 7x 10⁷ purified murine B cells were incubated with
LPS and protein H for 24 hours. Medium (1), cytoplasmic
material (2), nuclear extract (3), and protein H (4) were
run on SDS-PAGE and stained with Coomassie (left) or
5   blotted to a PVDF membrane (right). The blotted membrane
was probed with an anti-protein H antiserum, followed by
peroxidase-conjugated protein A and developed with ECL.
(B) Inhibition of proliferation of murine splenic B cells
in response to proteins H and A.

10

Figure 7. Schematic representation of protein H.

Sequence Information

15   Given below are the cDNA (SEQ ID No.5) and amino acid
(SEQ ID No.6) sequences of protein H. The boundaries of
the S, A, C1, C2, C3 and D regions are marked, as are the
N-termini of the alternative forms of protein H.

20   ATG ACT AGA CAA CAA ACC AAG AAA
                                              Met Thr Arg Gln Gln Thr Lys Lys
                                              -41
                                              Signal peptide (Region S)

25   AAT TAT TCA CTA CGG AAA CTA AAA ACC GGT ACG GCT TCA GTA GCC GTT
     Asn Tyr Ser Leu Arg Lys Leu Lys Thr Gly Thr Ala Ser Val Ala Val

     GCT TTG ACC GTT TTG GGC GCA GGT TTT GCA AAC CAA ACA ACA GTT AAG
     Ala Leu Thr Val Leu Gly Ala Gly Phe Ala Asn Gln Thr Thr Val Lys
30
     GCG|GAA GGG GCT AAA ATT GAT TGG CAA GAA GAG TAT AAA AAG TTA GAC
     Ala|Glu Gly Ala Lys Ile Asp Trp Gln Glu Glu Tyr Lys Lys Leu Asp
     -1 |1
         Mature protein, Region A
35
     GAA GAT AAT GCT AAA CTT GTT GAG GTT GTT GAA ACC ACA AGT TTG GAA
     Glu Asp Asn Ala Lys Leu Val Glu Val Val Glu Thr Thr Ser Leu Glu

     AAC GAA AAA CTC AAG AGT GAG AAT GAG GAG AAT AAG AAA AAT TTA GAC
40   Asn Glu Lys Leu Lys Ser Glu Asn Glu Glu Asn Lys Lys Asn Leu Asp

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         AAA CTT AGC AAA GAA AAT CAA GGA AAG CTC GAA AAA TTG GAG CTT GAC
         Lys Leu Ser Lys Glu Asn Gln Gly Lys Leu Glu Lys Leu Glu Leu Asp


         TAT CTC AAA AAA TTA GAT CAC GAG CAC AAA GAG CAC CAA AAA GAA CAA
   5     Tyr Leu Lys Lys Leu Asp His Glu His Lys Glu His Gln Lys Glu Gln


         CAA|GAA CAA GAA GAG CGA CAA AAA AAT CAA GAA CAA TTA GAA CGT AAA
         Gln|Glu Gln Glu Glu Arg Gln Lys Asn Gln Glu Gln Leu Glu Arg Lys
         80 |81
  10        Region B


         TAC CAA CGA GAA GTA GAA AAA CGT TAT CAA GAA CAA CTC CAA AAA CAA
         Tyr Gln Arg Glu Val Glu Lys Arg Tyr Gln Glu Gln Leu Gln Lys Gln


  15


         CAA CAA TTA GAA ACA GAA|AAG CAA ATC TCA GAA GCT AGT CGT AAG AGC
         Gln Gln Leu Glu Thr Glu|Lys Gln Ile Ser Glu Ala Ser Arg Lys Ser
                            117|118
  20                          Region C1


         CTA AGC CGT GAC CTT GAA GCG TCT CGT GCA GCT AAA AAA GAC CTT GAA
         Leu Ser Arg Asp Leu Glu Ala Ser Arg Ala Ala Lys Lys Asp Leu Glu


  25     GCT GAG CAC CAA AAA CTT GAA GCT GAG CAC CAA AAA CTT AAA GAA|GAC
         Ala Glu His Gln Lys Leu Glu Ala Glu His Gln Lys Leu Lys Glu|Asp
                                                                158|159
                                                              Region C2


  30     AAA CAA ATC TCA GAC GCA AGT CGT CAA GGC CTA AGC CGT GAC CTT GAA
         Lys Gln Ile Ser Asp Ala Ser Arg Gln Gly Leu Ser Arg Asp Leu Glu


         GCG TCT CGT GCA GCT AAA AAA GAG CTT GAA GCA AAT CAC CAA AAA CTT
         Ala Ser Arg Ala Ala Lys Lys Glu Leu Glu Ala Asn His Gln Lys Leu


  35
         GAA GCT GAG CAC CAA AAA CTT AAA GAA|GAC AAA CAA ATC TCA GAC GCA
         Glu Ala Glu His Gln Lys Leu Lys Glu|Asp Lys Gln Ile Ser Asp Ala
                                        200|201
                                       Region C3

  40
         AGT CGT CAA GGC CTA AGC CGT GAC CTT GAA GCG TCT CGT GCA GCT AAA
         Ser Arg Gln Gly Leu Ser Arg Asp Leu Glu Ala Ser Arg Ala Ala Lys


         AAA GAG CTT GAA GCA AAT CAC CAA AAA CTT GAA GCA GAA GCA AAA GCA
  45     Lys Glu Leu Glu Ala Asn His Gln Lys Leu Glu Ala Glu Ala Lys Ala
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     CTC AAA GAA|CAA TTA GCG AAA CAA GCT GAA GAA CTT GCA AAA CTA AGA
     Leu Lys Glu|Gln Leu Ala Lys Gln Ala Glu Glu Leu Ala Lys Leu Arg
            242|243
                 Region D
5

     GCT GGA AAA GCA TCA GAC TCA CAA ACC CCT GAT ACA AAA CCA GGA AAC
     Ala Gly Lys Ala Ser Asp Ser Gln Thr Pro Asp Thr Lys Pro Gly Asn


     AAA GCT GTT CCA GGT AAA GGT CAA GCA CCA CAA GCA GGT ACA|AAA CCT
10   Lys Ala Val Pro Gly Lys Gly Gln Ala Pro Gln Ala Gly Thr|Lys Pro
                                                        285|286
     Approx C-terminus of protein H cleaved from S. pyogenes|


     AAC CAA AAC AAA GCA CCA ATG AAG GAA ACT AAG AGA CAG TTA CCA TCA
15   Asn Gln Asn Lys Ala Pro Met Lys Glu Thr Lys Arg Gln Leu Pro Ser
                                        C-terminus of protein H produced in


     ACA GGT|GAA ACA GCT AAC CCA TTC TTC ACA GCG GCA GCC CTT ACT GTT
     Thr Gly|Glu Thr Ala Asn Pro Phe Phe Thr Ala Ala Ala Leu Thr Val
20           305|306
     E. Coli|


     ATG GCA ACA GCT GGA GTA GCA GCA GTT GTA AAA CGC AAA GAA GAA AAC
     Met Ala Thr Ala Gly Val Ala Ala Val Val Lys Arg Lys Glu Glu Asn
25                                                              335
                          C-terminus of full, mature protein H
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CLAIMS

1.   A nuclear delivery construct comprising:

5   (i)   protein H or a fragment or derivative thereof that
              is capable of being targeted to the nucleus of a
              eukaryotic cell; and

              associated therewith

10
     (ii)   one or more other components whose targeting to the
              nucleus of the eukaryotic cell is desired.

2.   A nuclear delivery construct according to claim 1
15   wherein the fragment or derivative of protein H
              comprises the A region of protein H, or a fragment
              or derivative thereof that is capable of being
              targeted to the nucleus of a eukaryotic cell.

20   3.   A nuclear delivery construct according to claim 2
              wherein the fragment or derivative of protein H
              comprises the AB region of protein H, or a
              derivative thereof that is capable of being targeted
              to the nucleus of a eukaryotic cell.

25
     4.   A nuclear delivery construct according to any one of
              the preceding claims comprising, as component (i), a
              fragment or derivative of protein H which is capable
              of being targeted to the nucleus of a eukaryotic
30   cell but does not have a cytostatic effect on the
              cell.

     5.   A nuclear delivery construct according to any one of
              the preceding claims wherein component (ii) is a

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polypeptide, nucleic acid, small molecule drug or a liposome.

6.   A nuclear delivery construct according to claim 5
     wherein component (ii) is a polypeptide and the
     construct is in the form of a fusion protein.

7.   A nuclear delivery construct according to any one of
     claims 1 to 6 wherein components (i) and (ii) are
     covalently or non-covalently associated.

8.   A pharmaceutical composition comprising a nuclear
     delivery construct as defined in any one of the
     preceding claims; and a pharmaceutically acceptable
     carrier.

9.   A nuclear delivery construct as defined in any one
     of claims 1 to 7 for use in a method of treatment of
     the human or animal body, or for use in a method of
     diagnosis.

10. Use of protein H or a fragment or derivative thereof
     that is capable of being targeted to the nucleus of
     a eukaryotic cell in the manufacture of a medicament
     for the treatment of the human or animal body in
     which a construct as defined in any one of claims 1
     to 7 is targeted to the nucleus of a eukaryotic
     cell.

11. Use of a nuclear delivery construct as defined in
     any one of claims 1 to 7 in the manufacture of a
     medicament for the treatment of disease of the human
     or animal body in which the construct is targeted to
     the nucleus of a eukaryotic cell.

12. Use according to claim 10 or 11 wherein the
     construct is targeted to the nucleus of a cell in
     which nucleophosmin (NPM)/B23 is up-regulated.

13. Use according to claim 10, 11 or 12 wherein
     component (i) of the construct interacts with actin,
     NPM/B23, protein SET or hnRNP A2/B1.

14. Use according to any one of claims 11 to 13 wherein
     the cell is a tumour cell or a proliferating cell.

15. Use according to any one of claims 10 to 14 wherein
     the cell is a virus-infected cell.

16. Use according to any one of claims 10 to 15 wherein
     the cell is a lymphocyte.

17. Use according to any one of claims 10 to 16 wherein
     component (ii) of the construct is a nucleic acid
     encoding a protein, and targeting to the nucleus
     effects gene therapy of the cell.

18. A nucleic acid sequence encoding a fusion protein
     according to claim 6.

19. A vector comprising a nucleic acid sequence
     according to claim 18.

20. A cell comprising a nucleic acid sequence according
     to claim 18 or a vector according to claim 19.

21. A method of producing a fusion according to claim 6
     comprising expressing a nucleic acid sequence
     according to claim 18 in a cell, and recovering the
     fusion protein thus obtained.

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22. A method of treating a disease of the human or
animal body by targeting a construct as defined in
any one of claims 1 to 7 to the nucleus of a
eukaryotic cell, comprising administering an

5   effective amount of the construct to a human or
animal subject.

23. A method according to claim 18 wherein the disease
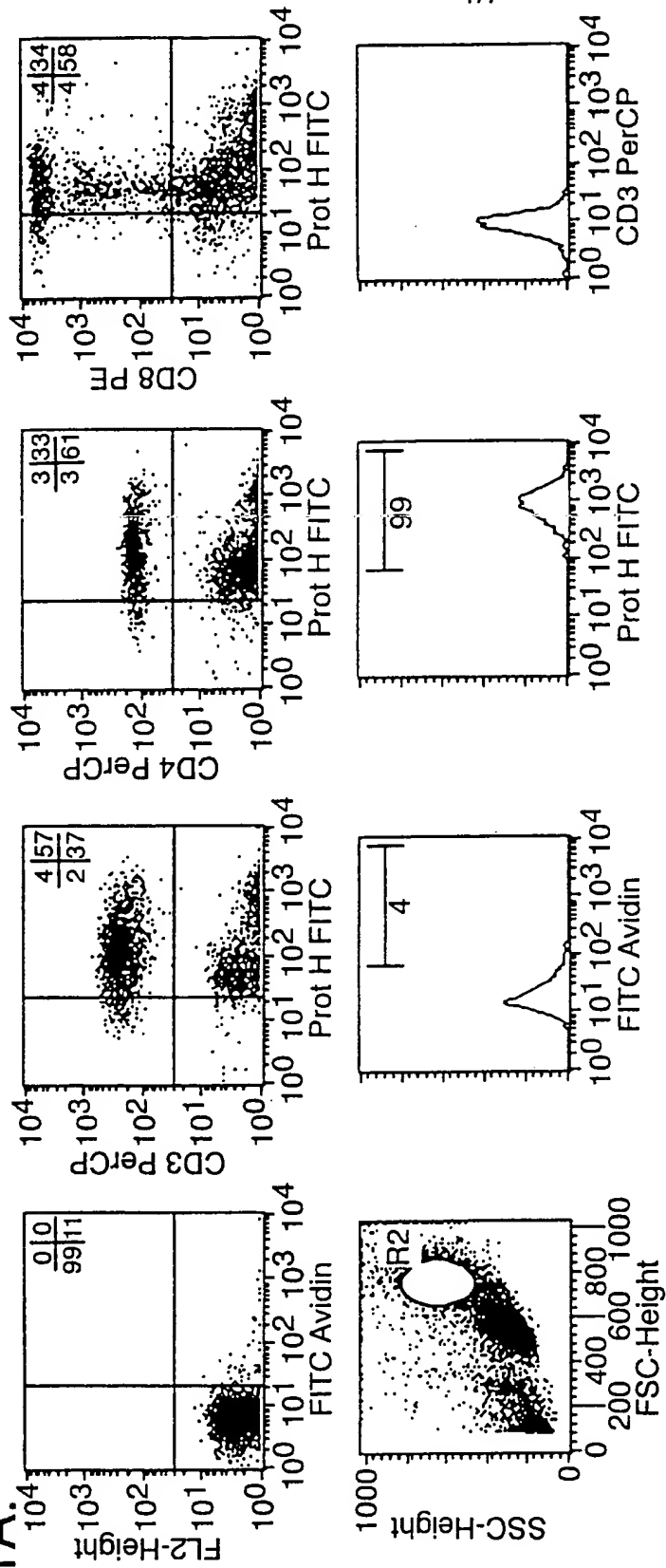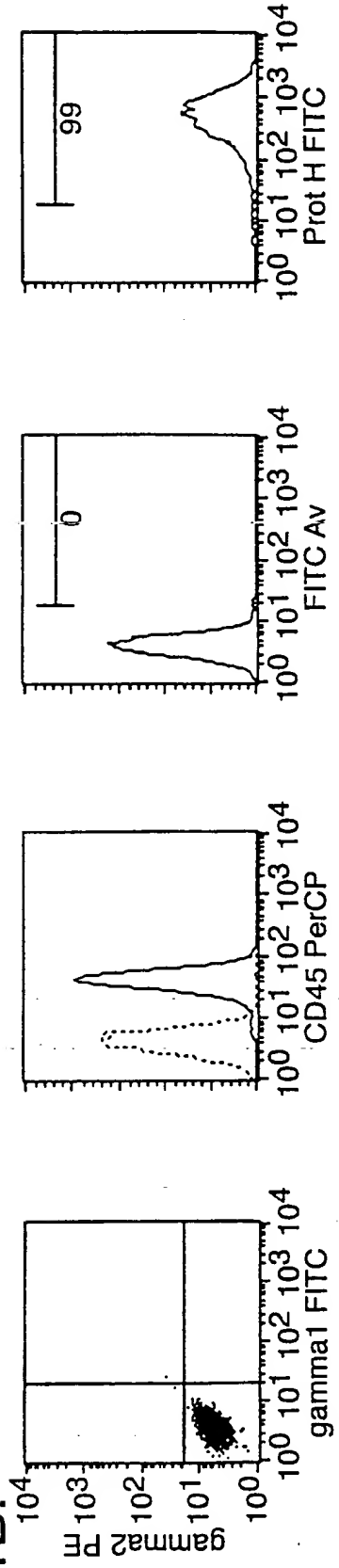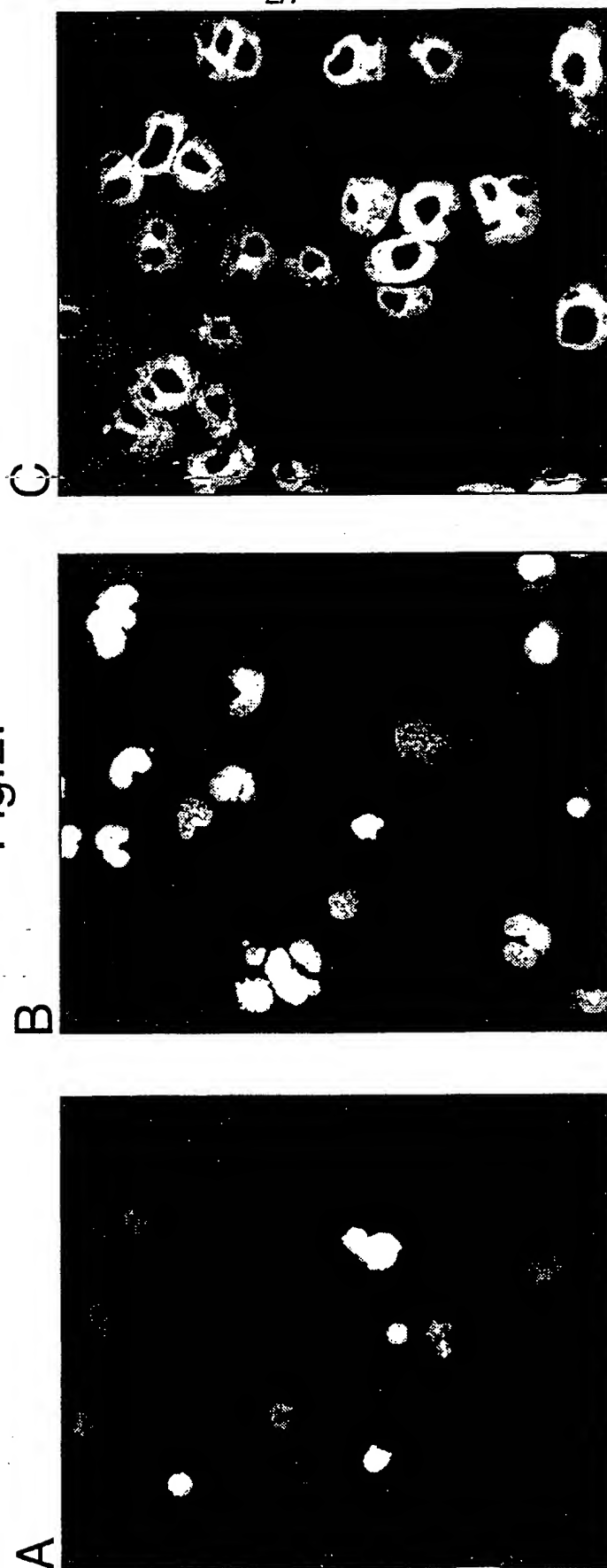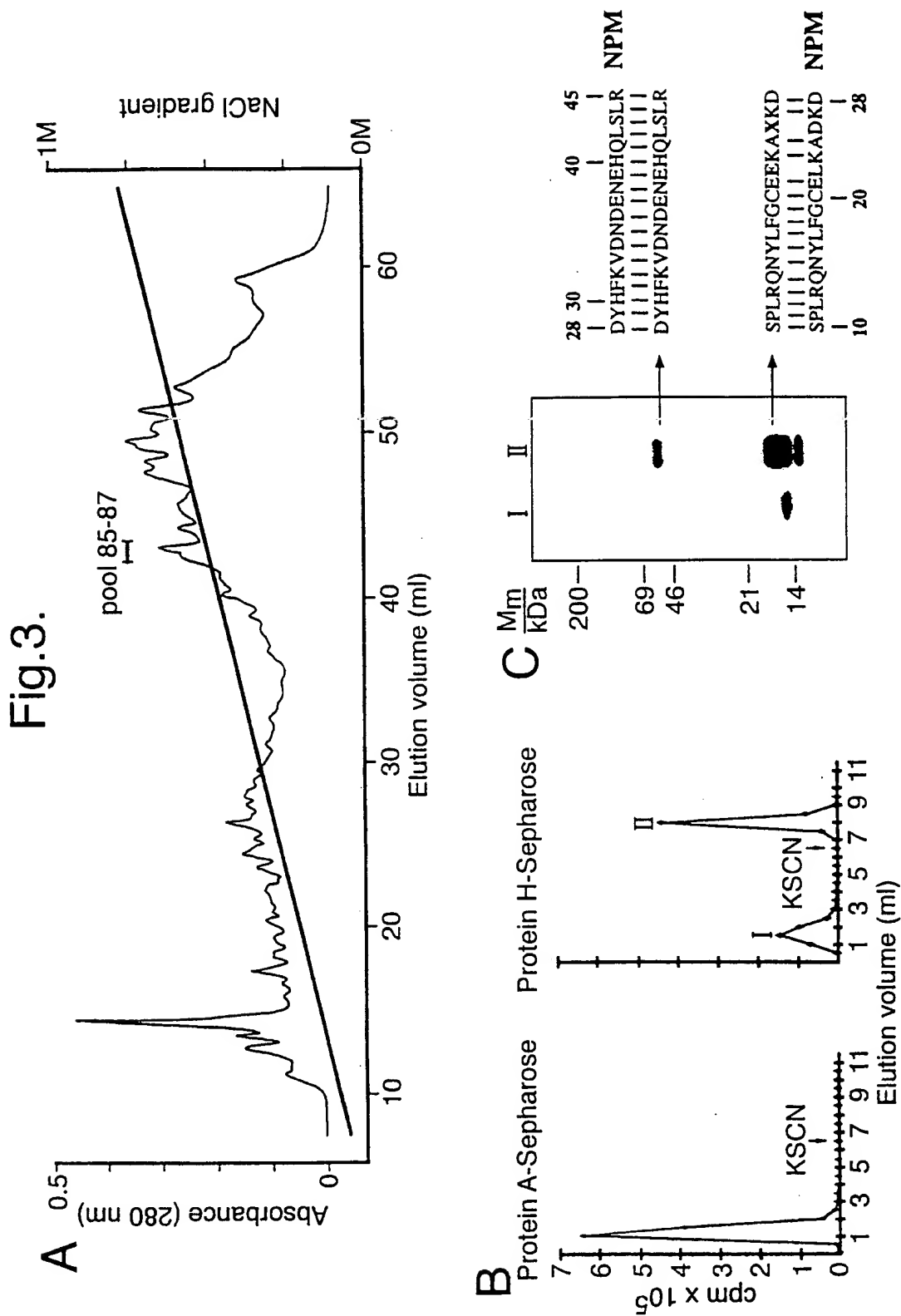is a tumour or a viral infection.

10

Fig.1A.

Fig.1B.

Fig.2.

Fig.3.

Fig.3 (Cont).

Fig.4.

	63	70	110	119	124	137
SET	LRQPFFQK		VEVTEFEDIK		IDFYFDENPYFENK	
Band I	LRQPFFQK		VEVTEFEDIK		IDFYFDENPYFENK	

	154	160	214	222
hnRNP A2/B1	GFGFVTF		GGGGNFGPG	
Band II	GFGFVTF		GGGGNFGPG	

	110	119	124	135	142	151
SET	VEVTEFEDIK		IDFYFDENPYFE		EFHLNESGDP	
Band III	VEVTEFEDIK		IDFYFDENPYFE		EFHLNESGDP	

Fig.5.

Fig.6.

A

B